

# Morphological and molecular analyses reveal two new species of *Microcera* (Nectriaceae, Hypocreales) associated with scale insects on walnut in China

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## Abstract

The fungal genus *Microcera* consists of species mostly occurring as parasites of scale insects, but are also commonly isolated from soil or lichens. In the present study, we surveyed the diversity and assess the taxonomy of entomopathogenic fungi in Sichuan Province, China. Two new species of *Microcera*, viz. *M. chrysomphaludis* and *M. pseudaulacaspidis*, were isolated from scale insects colonising walnut (*Juglans regia*). Maximum Likelihood and Bayesian Inference analyses of ITS, LSU, *tef1*-α, *rpb1*, *rpb2*, *act1*, *act*, *tub2*, *cmdA* and *his3* sequence data provide evidence for the validity of the two species and their placement in Nectriaceae (Hypocreales). *Microcera pseudaulacaspidis* primarily differs from similar species by having more septate and smaller cylindrical macroconidia, as well as DNA sequence data. Meanwhile, *Microcera chrysomphaludis* has elliptical, one-septate ascospores with acute ends and cylindrical, slightly curved with 4–6 septate macroconidia up to 78 μm long. Morphological descriptions with illustrations of the novel species and DNA-based phylogeny generated from analyses of multigene dataset are also provided to better understand species relationships.

**Key words:** Two new taxa, entomopathogenic fungi, morphology, phylogenetic analyses



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## Introduction

The genus *Microcera* Desm. (Nectriaceae, Hypocreales) was introduced in the 19<sup>th</sup> century and was typified by *M. coccophila* Desm., commonly known as the “red-headed fungus”. *Microcera* has been considered to be a synonym of the *Fusarium* Link in some major taxonomic revisions (Booth 1971; Nelson et al. 1983; Leslie and Summerell 2006). The genus is characterised by superficial, flame-like conidiomata, forming a fusarium-like asexual stage (Gräfenhan et al. 2011; Herrera et al. 2013). *Microcera* species exhibit diverse ecological characteristics and are typically regarded as entomogenous fungi that are associated

with scale insects, although they can occasionally be isolated from other substrates, such as aphids, adelgids, lichens and soil (Gräfenhan et al. 2011; Crous et al. 2021a, b, 2022a).

Currently, there are eight accepted species within the genus *Microcera* (Bills et al. 2009; Gräfenhan et al. 2011; O'Donnell et al. 2012; Herrera et al. 2013; Dao et al. 2015, 2016; Lombard et al. 2015; Crous et al. 2021b, 2022a; Xu et al. 2021). Based on DNA sequence data and ecological association, Gräfenhan et al. (2011) revised many anamorph- and teleomorph-typified genera of the Nectriaceae, resurrected *Microcera* and accepted four *Microcera* species, viz., *M. coccophila*, *M. diploa* (Berk. & M.A. Curtis) Gräfenhan & Seifert, *M. rubra* Gräfenhan & Seifert and *M. larvarum* (Fuckel) Gräfenhan, Seifert & Schroers. Lombard et al. (2015) supported *Microcera* as a monophyletic group distantly related to *Fusarium*, based on further phylogenetic inferences from DNA sequence data. Xu et al. (2021) isolated *M. kuwanaspidis* X.L. Xu & C.L. Yang from armoured scale insects *Kuwanaspis howardi* on *Phyllostachys heteroclada* in China. Two additional species, *M. lichenicola* and *M. physciae* Crous & Boers have been described from lichens (Crous et al. 2021b, 2022a).

During a survey of entomopathogenic fungi in Sichuan Province, China, two *Microcera* species, in association with the two scale insects *Pseudaulacaspis pentagona* and *Chrysomphalus aonidum* on walnut, were isolated. *Microcera pseudaulacaspidis* sp. nov. and *M. chrysomphaludis* sp. nov. are introduced here based on the morphological characteristics and multi-locus analyses (DNA based). They were compared morphologically with existing taxa. In this study, comprehensive descriptions, micrographs of macroscopic and microscopic morphological characteristics, as well as DNA sequence data, are provided to support the establishment of the new species.

## Materials and methods

### Specimen collection and isolation

Three specimens of scale insects (SICAU 22-0161, SICAU 22-0162 and SICAU 22-0163) that were infected, were collected from Neijiang City (29°48'15"N, 105°06'44"E) and Liangshan Yi Autonomous Prefecture (26°56'43"N, 102°16'16"E), Sichuan Province, on 16 April and 8 October 2022. The specimens were placed in sterilised tubes or plastic boxes and returned to the laboratory as described by Senanayake et al (2020). The fungi were isolated, based on the single spore isolation technique described by Chomnunti et al. (2014). Cultures were grown on PDA for 20–40 days, at 25 °C, under 12 h light/12 h dark for recording growth rates, shape, texture and colour of the colonies. Ascomata and sporodochia were observed and photographed using a dissecting microscope NVT-GG (Shanghai Advanced Photoelectric Technology Co. Ltd., Shanghai, China). We observed microscopic characteristics, such as asci, ascospores, pseudoparaphyses, ascomata wall, conidia, conidiophores, number of septa, metulae and conidiophores using an Olympus BX43. No fewer than 20 measurements of the two species were made for each feature using the Image Frame Work (IFW 0.9.0.7). The type specimens were deposited at the Herbarium of Sichuan Agricultural University, Chengdu, China (SICAU). The ex-type cultures were deposited at the Culture Collection in Sichuan Agricultural University (SICAUCC).

DNA extraction, PCR amplification and nucleotide sequencing

The New Plant Genomic DNA Kit (Beijing Aidlab Biotechnologies Co., Ltd, Beijing, China) was used to extract genomic DNA from fresh fungal mycelium. The extracted DNA to be used was stored at -20 °C. Amplified gene markers and their corresponding primers are shown in Table 1. Polymerase chain reaction (PCR) was performed in 25 µl reaction mixture containing 22 µl Master Mix (Beijing LABLEAD Biotech Co., Ltd., Beijing, China), 1 µl DNA template and 1 µl each of forward and reverse (10 µM) primers. The amplification reactions were performed as described by Gräfenhan et al. (2011), Lombard et al. (2015), Dai et al. (2016) and Wanasinghe et al. (2021). PCR products were sequenced at Hangzhou Youkang Biotech Co., Ltd., Chengdu, China. The newly-generated sequences were deposited in GenBank. New species are established as recommended by Jeewon and Hyde (2016).

Sequence alignment and phylogenetic analyses

Based on BLAST searches in GenBank and recent publications (Bills et al. 2009; Gräfenhan et al. 2011; O'Donnell et al. 2012; Herrera et al. 2013; Dao et al. 2015, 2016; Lombard et al. 2015; Xu et al. 2021), using the large subunit of the ATP citrate lyase (*ac1*), actin (*act*) regions, calmodulin (*cmdA*), histone H3 (*his3*), the internal transcribed spacer (ITS), the partial large subunit nuclear rDNA (LSU), the RNA polymerase II largest subunit (*rpb1*), the RNA polymerase II second largest subunit (*rpb2*), translation elongation factor 1-alpha (*tef1-α*), β-tubulin (*tub2*) and sequence data, reference sequences were downloaded and separate phylogenetic analyses, based on single gene datasets were carried out to initially determine the placement of the two species. Information on the taxa used and GenBank

Table 1. Gene markers and primer pairs used in this study.

| Gene markers | Primers     | Sequences of Primers 5'-3' | References                    |
|--------------|-------------|----------------------------|-------------------------------|
| <i>ac1</i>   | ac1-230up   | AGCCCGATCAGCTCATCAAG       | Gräfenhan et al. (2011)       |
|              | ac1-1220low | CCTGGCAGCAAGATCVAGGAAGT    |                               |
| <i>act</i>   | ACT-512F    | ATGTGCAAGGCCGGTTTCGC       | Carbone and Kohn (1999)       |
|              | ACT1Rd      | CRTCCTACTCCTGCTTBGAGATCCAC | Groenewald et al. (2013)      |
| <i>cmdA</i>  | CAL-228F    | GAGTTCAAGGAGGCCTTCTCCC     | Carbone and Kohn 1999)        |
|              | CAL2Rd      | TGRTCNGCCTCDCGGATCATCTC    | Groenewald et al. (2013)      |
| <i>his3</i>  | CYLH3F      | AGGTCCACTGGTGGCAAG         | Crous et al. (2006)           |
|              | CYLH3R      | AGCTGGATG TCCTTGGAC        |                               |
| ITS          | ITS5        | GGAAGTAAAAGTCGTAACAAGG     | White et al. (1990)           |
|              | ITS4        | TCCTCCGCTTATTGATATGC       |                               |
| LSU          | LR0R        | ACCCGCTGAACTTAAGC          | Rehner and Samuels (1994)     |
|              | LR5         | ATCCTGAGGGAAACTTC          | Vilgalys and Hester (1990)    |
| <i>rpb1</i>  | RPB1-Ac     | CAYCCWGGYTTYATCAAGAA       | Castlebury et al. (2004)      |
|              | RPB1-Cr     | CCNGCDATNTCRRTRTCCATRTA    |                               |
| <i>rpb2</i>  | RPB2-5F2    | GGGGWGAYCAGAAGAAGGC        | O'Donnell et al. (2007)       |
|              | RPB2-7cR    | CCCATRGCTTGYTTRCCCAT       |                               |
| <i>tef1</i>  | EF1-728F    | CATCGAGAAGTTCGAGAAGG       | Carbone and Kohn (1999)       |
|              | EF2         | GGARGTACCAGTSATCATG        | O'Donnell et al. (1998)       |
| <i>tub2</i>  | T1          | AACATGCGTGAGATTGTAAGT      | O'Donnell and Cigelnik (1997) |
|              | CYLTUB1R    | AGTTGTCCG GACGGAAGAG       | Crous et al. (2006)           |

accession numbers of our novel species are listed in Table 2. Alignments for the individual locus matrices were generated with the online version of MAFFT version 7.429 (Kato and Standley 2013) and ambiguous regions were excluded using BioEdit version 7.0.5.3 (Hall 1999). Combined sequences of ITS, LSU, *tef1-α*, *rpb1*, *rpb2*, *ac1*, *act*, *tub2*, *cmdA* and *his3* were performed by SequenceMatrix v.1.7.8 (Vaidya et al. 2011). Maximum Likelihood (ML) and Bayesian Inference (BI) were constructed as described in Xu et al (2020). The phylogenetic tree constructed was viewed and edited using FigTree version 1.4.2 and Adobe Illustrator CS6.

### Genealogical concordance phylogenetic species recognition analysis

Phylogenetically closely-related species were analysed using the Genealogical Concordance Phylogenetic Species Recognition (GCPSR) model by performing a pairwise homoplasy index (PHI) test as described by Quaedvlieg et al. (2014). The PHI test was performed in SplitsTree v.4.17.1 (Huson 1998; Huson and Bryant 2006) in order to determine the recombination level within phylogenetically closely-related species using a 6-locus concatenated dataset (ITS, LSU, *tef1-α*, *ac1*, *cmdA* and *his3*). The results can be visualised by constructing a split graph using LogDet conversion and the Splits options. Pairwise homoplasy index below a 0.05 threshold ( $\Phi_w < 0.05$ ) indicates significant recombination present in the dataset. The relationship between closely-related species was visualised by constructing a Splits graph.

## Results

### Phylogenetic analyses

The ML and BI analyses resulted in trees with similar topologies. Multi-locus phylogenetic analyses of species of Nectriaceae (Hypocreales) include sequences from 25 taxa and *Tilachlidium brachiatum* (Batsch) Petch (CBS 363.97, CBS 505.67) were used as outgroup (Fig. 1). The alignment contained 11882 characters (ITS = 1213, LSU = 1456, *tef1-α* = 1246, *rpb1* = 1634, *rpb2* = 2053, *ac1* = 1060, *act* = 1206, *tub2* = 707, *cmdA* = 779, *his3* = 530), including gaps. The matrix had 4402 distinct alignment patterns, with 51.12% of undetermined characters or gaps. Estimated base frequencies were as follows: A = 0.236233, C = 0.270063, G = 0.255057, T = 0.238647, with substitution rates AC = 1.239837, AG = 3.452130, AT = 1.264349, CG = 0.971857, CT = 5.853200 and GT = 1.000000. The gamma distribution shape parameter  $\alpha = 0.347827$  and the Tree-Length = 2.637211. The best scoring RAxML tree with a final likelihood value of -68,438.855836 is presented in Fig. 1 where the isolates from this study formed two distinct, well-supported lineages (MLBS = 100%, BIPP = 1.00) and, thus, were considered to represent two previously-unknown species.

### Pairwise homoplasy index (PHI) test

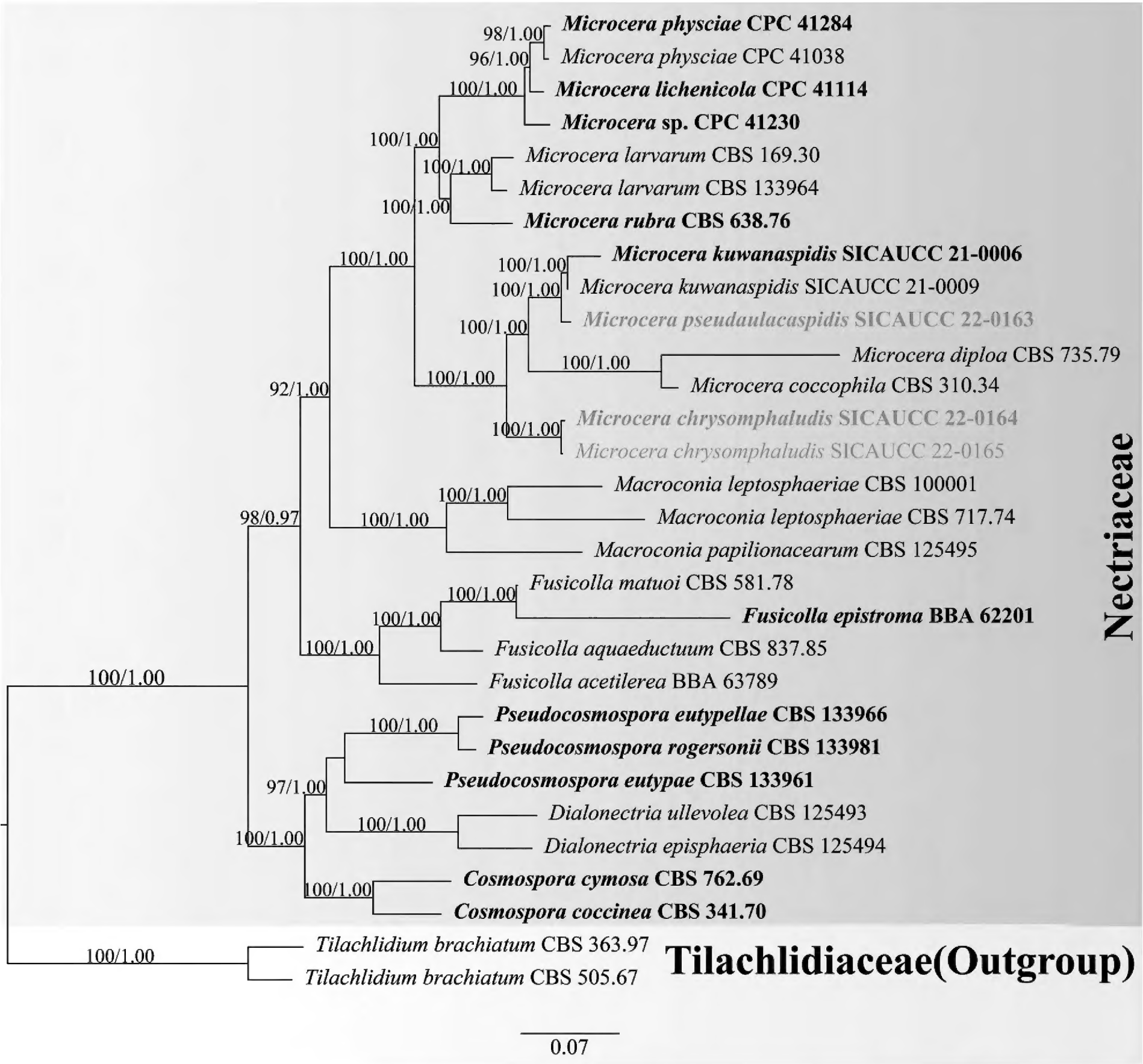
The pairwise homoplasy index (PHI) test revealed that there was no significant recombination ( $\Phi_w = 1$ ) between *Microcera pseudaulacaspidis* (SICAUCC 22-0163), *M. coccophila* (CBS 310.34), *M. diploa* (CBS 735.79) and *M. kuwanaspidis* (SICAUCC 21-0006) (Fig. 2).



Table 2. Specimen information and GenBank accession numbers of the sequences used in this study.

| Species                                   | Strain/Voucher No.  | GenBank Accession No. |                 |                 |                 |                 |                 |                 |                 |                 |                 |
|---|---|-----------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|   |   | acI1                  | act             | cmdA            | his3            | ITS             | LSU             | rpb1            | rpb2            | tef I-α         | tub2            |
| <i>Cosmospora coccinea</i>                | CBS 341.70 <sup>†</sup>   | HQ897913              | KM231221        | KM231398        | KM231550        | HQ897827        | KM231692        | KM232242        | HQ897777        | KM231947        | KM232086        |
| <i>Cosmospora cymosa</i>                  | CBS 762.69 <sup>†</sup>   | HQ897914              | KM231222        | KM231399        | KM231551        | HQ897828        | KM231693        | KM232243        | HQ897778        | KM231948        | KM232087        |
| <i>Dialonectria episphaeria</i>           | CBS 125494 = TG 2006-11   | HQ897892              | KM231227        | KM231404        | KM231556        | HQ897811        | KM231697        | KM232248        | HQ897756        | KM231953        | KM232092        |
| <i>Dialonectria ullevolea</i>             | CBS 125493 = TG 2007-56   | HQ897918              | KM231226        | KM231403        | KM231555        | KM231821        | KM231696        | KM232247        | HQ897782        | KM231952        | KM232091        |
| <i>Fusicolla acetilerea</i>               | BBA 63789 <sup>†</sup> = IMI181488 = NRRL20827                  | KM231065              | -               | -               | -               | HQ897790        | U88108          | -               | HQ897701        | -               | -               |
| <i>Fusicolla aquaeductuum</i>             | CBS 837.85 = BBA 64559 = NRRL 20865                             | KM231067              | -               | KM231406        | -               | KM231823        | KM231699        | KM232250        | HQ897744        | KM231955        | KM232094        |
| <i>Fusicolla epistroma</i>                | BBA 62201 <sup>†</sup> = IMI 85601 = NRRL 20439 = KNUF-20-PBU01 | KM231069              | -               | -               | -               | OW982703        | AF228352        | LC592349        | HQ897765        | -               | -               |
| <i>Fusicolla matuoi</i>                   | CBS 581.78 = ATCC 18694 = MAFF 238445 = NRRL 20427              | KM231070              | KM231228        | KM231405        | KM231557        | KM231822        | KM231698        | KM232249        | HQ897720        | KM231954        | KM232093        |
| <i>Macroconia leptosphaeriae</i>          | CBS 717.74  | KM231062              | KM231236        | KM231414        | KM231564        | KM231827        | KM231707        | KM232257        | KM232390        | JF735695        | KM232099        |
| <i>Macroconia leptosphaeriae</i>          | CBS 100001 = CBS H-6030   | KM231063              | KM231234        | KM231412        | KM231562        | HQ897810        | KM231705        | KM232255        | HQ897755        | KM231959        | KM232097        |
| <i>Macroconia papilionacearum</i>         | CBS 125495  | HQ897912              | KM231233        | KM231411        | KM231561        | HQ897826        | KM231704        | KM232254        | HQ897776        | KM231958        | KM232096        |
| <b><i>Microcera chrysomphaludis</i></b>   | <b>SICAUCC 22-0164<sup>†</sup></b>                              | <b>OQ569756</b>       | <b>OQ569739</b> | <b>OQ599375</b> | <b>OQ569753</b> | <b>OQ434281</b> | <b>OQ434276</b> | <b>OQ569747</b> | <b>OQ569742</b> | <b>OQ438144</b> | <b>OQ569750</b> |
| <b><i>Microcera chrysomphaludis</i></b>   | <b>SICAUCC 22-0165</b>  | <b>OQ569757</b>       | <b>OQ569740</b> | <b>OQ599376</b> | <b>OQ569754</b> | <b>OQ434282</b> | <b>OQ434277</b> | <b>OQ569748</b> | <b>OQ569743</b> | <b>OQ438145</b> | <b>OQ569751</b> |
| <i>Microcera coccophila</i>               | CBS 310.34 <sup>†</sup> = NRRL 13962 = G.J.S. 98-50             | HQ897843              | KM231232        | KM231410        | KM231560        | MH855540        | KM231703        | JX171462        | JX171576        | JF740692        | KC291937        |
| <i>Microcera diploa</i>                   | CBS 735.79 = BBA 62173 = NRRL 13966 = NRRL 36545                | HQ897899              | -               | -               | -               | HQ897817        | MW827663        | JX171463        | HQ897763        | JF740693        | -               |
| <i>Microcera kuwanaspidis</i>             | SICAUCC 21-0006 <sup>†</sup>                                    | MW462125              | MW462126        | MW462127        | MW462128        | MW484993        | MW462905        | MW462129        | MW462124        | MW462117        | MW462130        |
| <i>Microcera kuwanaspidis</i>             | SICAUCC 21-0009   | MZ044037              | MZ044038        | MZ044039        | MZ044040        | MZ029437        | MZ029436        | MZ044041        | MZ044036        | MZ044035        | MZ044042        |
| <i>Microcera larvarum</i>                 | CBS 169.30  | HQ897855              | -               | -               | EU860049        | EU860064        | EU860064        | -               | HQ897717        | -               | EU860025        |
| <i>Microcera larvarum</i>                 | A.R. 4580 = CBS 133964  | -                     | -               | -               | -               | KC291751        | KC291759        | KC291894        | -               | KC291832        | KC291935        |
| <i>Microcera lichenicola</i>              | CPC 41114 <sup>†</sup> = CBS 149169                             | -                     | -               | -               | -               | ON811502        | ON811561        | -               | -               | -               | ON803591        |
| <i>Microcera physciae</i>                 | CPC 41284 = CBS 148283  | -                     | -               | -               | -               | OK664727        | OK663766        | OK651153        | OK651168        | OK651190        | OK651208        |
| <i>Microcera physciae</i>                 | CPC 41038 <sup>†</sup> = CBS 148288                             | -                     | -               | -               | -               | OK664728        | OK663767        | OK651154        | OK651169        | OK651191        | OK651209        |
| <b><i>Microcera pseudaulacaspidis</i></b> | <b>SICAUCC 22-0163<sup>†</sup></b>                              | <b>OQ569755</b>       | <b>OQ569738</b> | <b>OQ599374</b> | <b>OQ569752</b> | <b>OQ434280</b> | <b>OQ434275</b> | <b>OQ569746</b> | <b>OQ569741</b> | <b>OQ438143</b> | <b>OQ569749</b> |
| <i>Microcera rubra</i>                    | CBS 638.76 <sup>†</sup> = BBA 62460 = NRRL 20475                | HQ897903              | KM231231        | KM231409        | EU860050        | HQ897820        | KM231702        | KM232253        | HQ897767        | JF740696        | EU860018        |
| <i>Microcera</i> sp.                      | CPC 41230 = CBS 148313  | -                     | -               | -               | -               | ON811503        | ON811562        | ON803533        | ON803543        | ON803570        | ON803592        |
| <i>Pseudocosmospora eutypae</i>           | CBS 133966 <sup>†</sup> =A.R.4562                               | -                     | -               | -               | -               | KC291721        | KC291757        | KC291871        | -               | KC291830        | KC291912        |
| <i>Pseudocosmospora eutypellae</i>        | C.H. 11-01 = CBS 133961 <sup>†</sup>                            | -                     | -               | -               | -               | KC291735        | KC291766        | KC291884        | -               | KC291837        | KC291925        |
| <i>Pseudocosmospora rogersonii</i>        | CBS 133981 <sup>†</sup> = G.J.S. 90-56                          | -                     | -               | -               | -               | KC291729        | KC291780        | KC291878        | -               | KC291852        | KC291915        |
| <i>Tilachlidium brachiatum</i>            | CBS 505.67  | KM231076              | KM231249        | KM231436        | -               | KM231839        | KM231720        | KM232272        | KM232415        | KM231976        | KM232110        |
| <i>Tilachlidium brachiatum</i>            | CBS 363.97  | KM231077              | KM231248        | KM231435        | KM231583        | KM231838        | KM231719        | KM232271        | KM232414        | KM231975        | KM232109        |

Notes: superscript T represents ex-type or ex-epitype isolates. “-” means that the sequence is missing or unavailable. New sequences are listed in bold



**Figure 1.** Phylogram generated from RAxML analysis, based on combined ITS, LSU, *tef1*-α, *rpb1*, *rpb2*, *ac1*, *act*, *tub2*, *cmdA* and *his3* sequence data of *Microcera* isolates. Bootstrap support values from Maximum Likelihood (MLBS, left) higher than 75% and Bayesian posterior probabilities (BIPP, right) equal to or greater than 0.95 are indicated at the nodes, respectively. The sequences from ex-type strains are in bold. The newly-generated sequence is in red.

### Taxonomy

***Microcera pseudaulacaspidis* Feng Liu & C.L. Yang, sp. nov.**

Index Fungorum No: 555034

Fig. 3

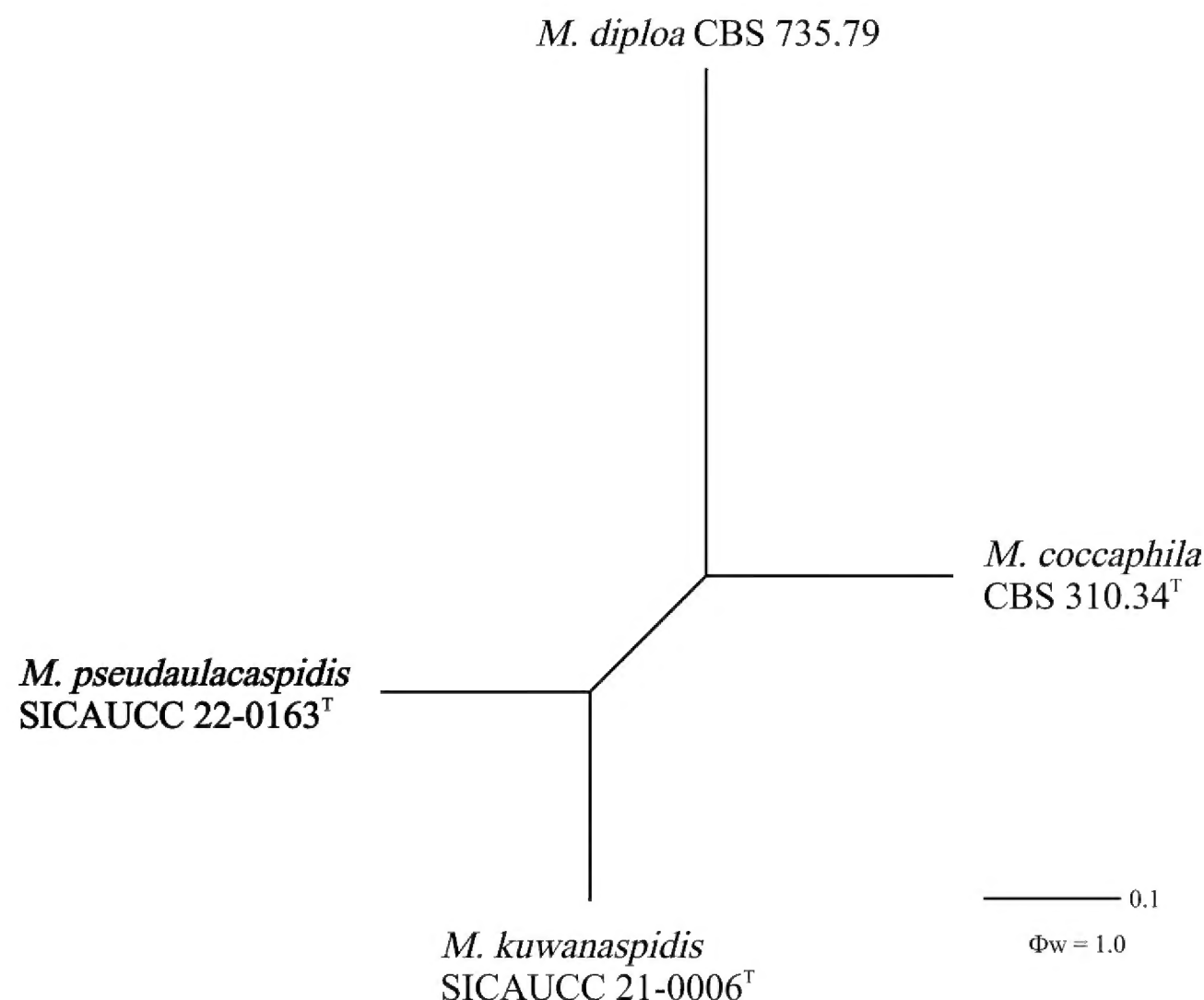
**Etymology.** In reference to the generic name of scale insect from which it was isolated.

**Holotype.** SICAU 22-0161.

**Host.** *Pseudaulacaspis pentagona* (Diaspididae, Homoptera)

**Habitat.** On the trunk of *Juglans regia*.

**Sexual state.** Undetermined.



**Figure 2.** The result of the pairwise homoplasy index (PHI) test of closely-related species using both LogDet transformation and Splits decomposition. PHI test results ( $\Phi_w$ ) < 0.05 indicate significant recombination within the dataset.

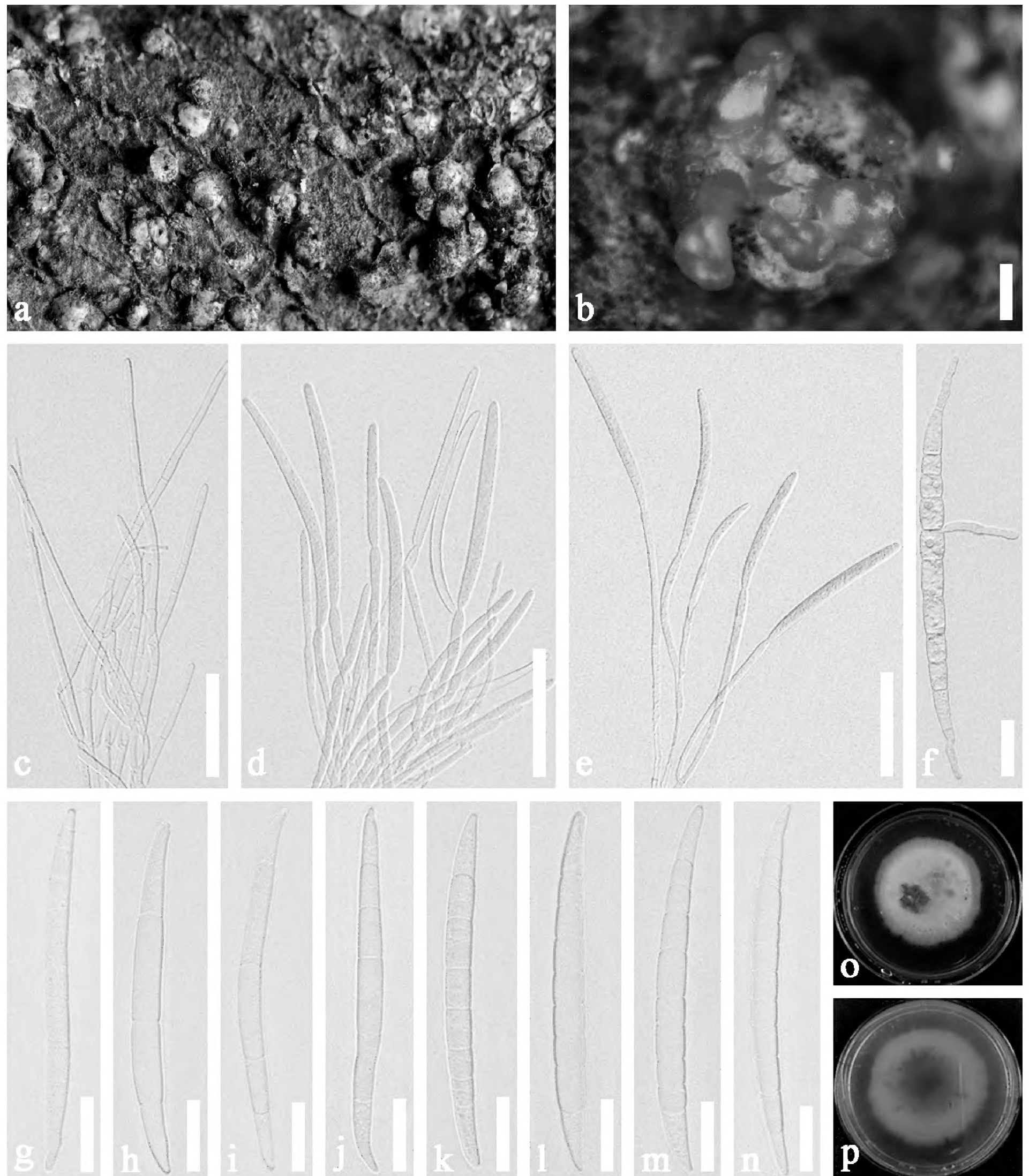
**Asexual state.** Stromata byssoid, well-developed, bright orange to orange-red, formed directly on the margin of host scales or their covers with 1–7 sporodochia. Sporodochia 250–900  $\mu\text{m}$  long, 400–860  $\mu\text{m}$  wide, ( $\bar{x}$  = 620  $\times$  570  $\mu\text{m}$ ,  $n$  = 50), conical, orange-red, upright masses on margin of host scales. Macroconidia 70–120  $\mu\text{m}$  long  $\times$  4.2–10.5  $\mu\text{m}$  wide ( $\bar{x}$  = 95.7  $\times$  6.5  $\mu\text{m}$ ,  $n$  = 50), hyaline or jasmine, cylindrical, slightly curved, slender towards each end, 3–10 septate, mostly 7–9 septate, difficult to distinguish apical cell and basal cell. Microconidia and chlamydospores were not observed.

**Material examined.** CHINA, Sichuan Province, Neijiang City, Dongxing District, Paifang Village walnut industrial base (29°48'15"N, 105°06'44"E, alt. 340 m), on scale insect *Pseudaulacaspis pentagona*, 16 April 2022, Feng Liu, LF202204001, (SICAU 22-0161, holotype), ex-type culture SICAUCC 22-0163.

**Culture characters.** Colonies from a single macroconidium on PDA grow slowly and reach approximately 2 cm in diameter after 12 days at 25 °C, circular, flat, producing masses of macroconidia in the centre of the colony, measuring 76–125  $\mu\text{m}$  long  $\times$  5.3–7.6  $\mu\text{m}$  wide ( $\bar{x}$  = 91.2  $\times$  6.3  $\mu\text{m}$ ,  $n$  = 50), smaller than those in nature, white mycelium on the surface and the back of colonies is dark orange.

**Notes.** Based on multi-gene phylogenetic analyses, *Microcera pseudaulacaspidis* is closely related to *M. kuwanaspidis* (Fig. 1). However, we observed significant differences in the DNA sequence data, including base-pair differences and gaps, with values of 1.45% (0 gaps), 17.67% (17 gaps), 3.22% (2 gaps), 1.53% (2 gaps), 1.70% (1 gap) and 3.82% (1 gap) in the ITS, LSU, *tef1*- $\alpha$ , *tub2*, *cmdA* and *his3* genes, respectively. The PHI test also showed that no significant recombination events between *M. pseudaulacaspidis* and closely phylogenetically-related species occurred (Fig. 2). Based on a comparison of their morphological characteristics, *M. pseudaulacaspidis* can be distinguished





**Figure 3.** *Microcera pseudaulacaspidis* (SICAU 22-0161) **a, b** stromata and sporodochia on host substrate **c–e** conidiophore with developing macroconidia **f** germinated conidium **g–o** Macroconidia **o, p** colonies on PDA after 30 days. Scale bars: 200 µm (**a, b**); 20 µm (**c–e**); 10 µm (**f–n**).

from *M. kuwanaspidis* by shorter macroconidia ( $95.7 \times 6.5 \mu\text{m}$  vs.  $107 \times 7.3 \mu\text{m}$ ) with more septa (7–9-septate vs. 5–7-septate) (Xu et al. 2021). Given the morphological dissimilarities, distinct nucleotides at various sites and the well-supported lineage in our phylogeny, we have sufficient evidence to establish *M. pseudaulacaspidis* as a new species.



***Microcera chrysomphaludis* Feng Liu & C.L. Yang, sp. nov.**

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**Etymology.** In reference to the generic name of scale insect from which it was isolated.

**Holotype.** SICAU 22-0162.

**Host.** *Chrysomphalus aonidum* (Diaspididae, Homoptera)

**Habitat.** On the trunk of *Juglans regia*.

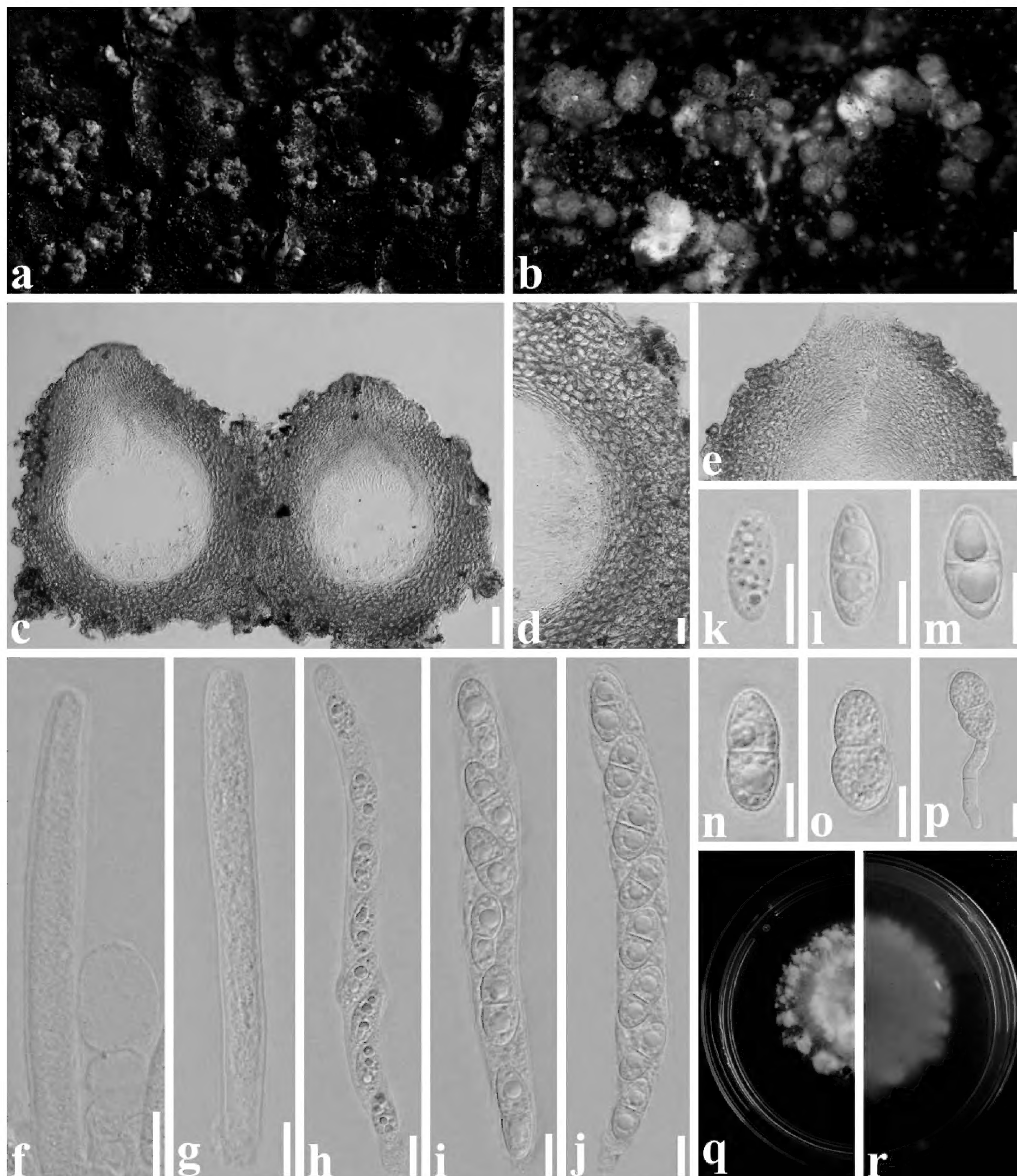
**Sexual state.** Perithecia 285–429  $\mu\text{m}$  high, 216–386  $\mu\text{m}$  diam. ( $\bar{x}$  = 350  $\times$  290  $\mu\text{m}$ ,  $n$  = 50), scattered, gregarious, formed directly on margin of host scales, bright red to dark red, subglobose, ellipsoidal in section, a central, rounded, papillate ostiole, lined internally with periphyses. Peridium 62–95  $\mu\text{m}$  thick, comprising two layers, outer stratum 32–55  $\mu\text{m}$  thick, composed of small, hyaline to light brown cells of textura angularis; inner stratum 35–45  $\mu\text{m}$  thick, composed of thinner, orange cells of textura angularis; thicker at sides towards apex, thinner at base. Hamathecium 8.5–19.2  $\mu\text{m}$  diameter ( $\bar{x}$  = 12.3  $\mu\text{m}$ ,  $n$  = 30), longer than asci, septate, unbranched, paraphyses. Asci 83.3–128.5  $\times$  7.5–15.2  $\mu\text{m}$  ( $\bar{x}$  = 109.2  $\times$  10.2  $\mu\text{m}$ ,  $n$  = 50), 8-spored, bitunicate, cylindrical, straight or curved, rounded at apex. Ascospores 16.8–27.5  $\times$  7.8–10.8  $\mu\text{m}$  ( $\bar{x}$  = 20.9  $\times$  9.6  $\mu\text{m}$ ,  $n$  = 50), uniseriate, elliptical, with rounded ends, one-septate, slightly constricted at septum, hyaline, smooth-walled, with many guttules.

**Asexual state.** Stromata byssoid, pale yellow, formed directly on margin of host scales with 1–6 sporodochia. Sporodochia conical, erupted, yellowish, scattered or aggregated. Macroconidia 73–89 long, 6.9–10.6  $\mu\text{m}$  wide ( $\bar{x}$  = 78.8  $\times$  8.5  $\mu\text{m}$ ,  $n$  = 50), hyaline, cylindrical, slightly curved, slender towards each end, 2–7 septa, mostly 4–6 septa, slightly constricted at septum, difficult to distinguish apical cell and basal cell. *Microconidia* and *chlamydospores* were not observed.

**Material examined.** CHINA, Sichuan Province, Liangshan Yi Autonomous Prefecture, Huili County (26°56'43"N, 107°16'16"E, alt. 1780 m), on scale insect *Chrysomphalus aonidum*, 8 October 2022, Feng Liu, LF202208001, (SICAU 22-0162, holotype), ex-type culture SICAUCC 22-0164. Ibid. LF202008002 (SICAU 22-0163, paratype), living culture SICAUCC 21-0165.

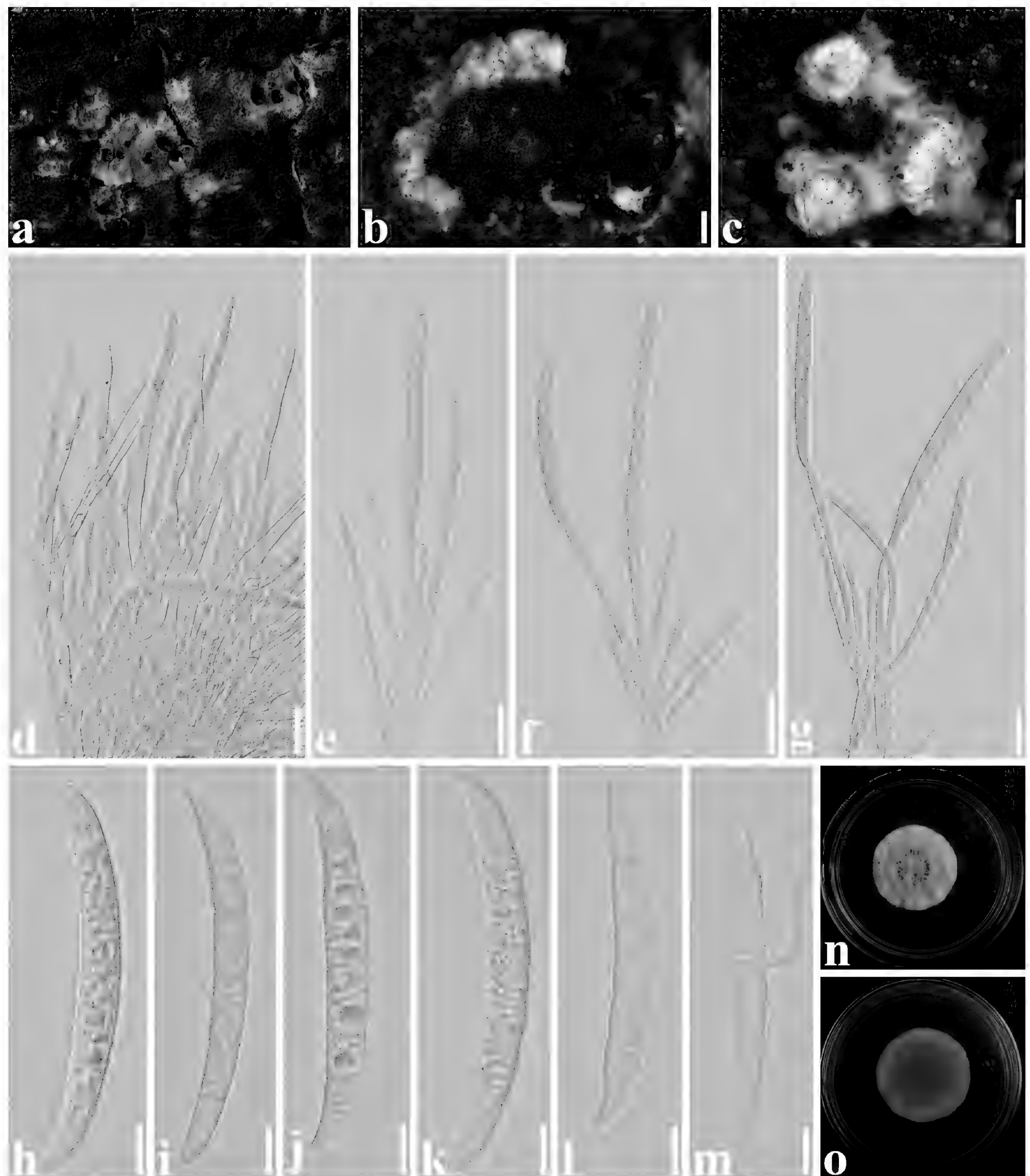
**Culture characters.** Ascospores germinate on PDA within 12 h and cultures grow slowly on PDA. Colonies reach 2.4 cm in diameter after 20 days. Colonies from single conidia flocculent, clinging to medium, with irregular margin, white to pink mycelium on surface and back of colonies dark orange. Mycelium creamy-white starting at centre, but gradually becoming pale pink after 20 days, forming sparsely distributed mycelial clumps near edge of colony. Conidia germinate on PDA within 12 h, cultures grow slowly on PDA. Colonies 2.5 cm in diameter after 20 days. Colonies from single ascospores cottony and hard, with regular margin; mycelium creamy-white to pale pink, with concentric rings; back of colonies pale yellow.

**Notes.** Multi-gene phylogenetic analyses have revealed that *Microcera chrysomphaludis* forms a highly robust clade that is closely related to *M. coccophila* and *M. diploa*. However, it is distinct from these two species with a high level of bootstrap support (ML/BY 100/1.00; Fig. 1). Morphologically, *M. chrysomphaludis* exhibits similar characteristics to *M. coccophila*, including superficial, subglobose, bright red ascomata, cylindrical asci and elliptical ascospores, as well as cylindrical macroconidia. However, *M. chrysomphaludis*



**Figure 4.** *Microcera chrysomphaludis* (SICAU 22-0162) **a, b** ascomata on host substrate **c** vertical section through ascotromata **d** peridium **e** ostiole of locule **f** paraphyses **h** ocular chamber **g–j** asci **k–o** ascospores **p** germinated ascospores; **q, r** colonies on PDA after 30 days. Scale bars: 200  $\mu\text{m}$  (**a, b**); 50  $\mu\text{m}$  **c**, 20  $\mu\text{m}$  (**d, e**); 10  $\mu\text{m}$  (**f–p**).

can be differentiated from *M. coccophila* by its larger ascomata (285–429  $\times$  216–386  $\mu\text{m}$  vs. 194–387  $\times$  194–355  $\mu\text{m}$ ), slightly shorter asci (109.2  $\times$  10.2  $\mu\text{m}$  vs. 115  $\times$  15  $\mu\text{m}$ ), longer ascospores (16.8–27.5  $\times$  7.8–10.8  $\mu\text{m}$  vs. 14–19  $\times$  6–10  $\mu\text{m}$ ) and shorter macroconidia (73–89  $\times$  6.9–10.6  $\mu\text{m}$  vs. 90–132  $\times$  6–9  $\mu\text{m}$ ) and fewer septa (4–6 vs. 7–9) (Gräfenhan et al. 2011; Dao et al. 2015). Hence, we describe our collection as a new species in *Microcera*.



**Figure 5.** *Microcera chrysomphaludis* (SICAU 22-0163) **a–c** stromata and sporodochia on host substrate **d–g** conidiophore with developing macroconidia **h–l** macroconidia **m** germinated conidium **n, o** colonies on PDA after 30 days. Scale bars: 200  $\mu\text{m}$  (**b, c**); 20  $\mu\text{m}$  (**d–g**); 10  $\mu\text{m}$  (**h–m**).

## Discussion

In this study, two new species (*Microcera chrysomphaludis* and *M. pseudaulacspidis*) associated with scale insects from walnut were introduced, based on phylogenetic inferences of a combined ITS, LSU, *tef1*- $\alpha$ , *ac11*, *act*, *cmdA*, *his3*, *rpb1*, *rpb2* and *tub2* DNA sequence dataset and morphological evidence.



Ecologically, *Microcera* species are mainly distributed in tropical regions, but they have also been reported in the subtropical and temperate regions. Most of the *Microcera* species are pathogens of scale insects (Gräfenhan et al. 2011; O'Donnell et al. 2012; Dao et al. 2015, 2016; Crous et al. 2021a; Xu et al. 2021). However, two new species have recently been described from lichens (Crous et al. 2021b, 2022a). Most *Microcera* species infecting scale insects occur in the tree canopy and are more noticeable under moist conditions (Dao et al. 2015, 2016; Xu et al. 2021), consistent with the findings of this study. Morphologically, the sexual morph in this genus is characterised by orange to dark red perithecia with a blunt papilla producing cylindrical to narrowly clavate asci and 1(–3)-septate ascospores, while the asexual morph is predominantly fusarium-like, with verticillate to penicillate conidiophores producing small macroconidia (Gräfenhan et al. 2011; Lombard et al. 2015; Crous et al. 2021a, b). Similar morphs were observed and documented in this study to provide further evidence of a connection between our isolates and other *Microcera* species (e.g. Figs 4, 5).

Gräfenhan et al. (2011) analysed an association of *Microcera* to *Fusarium*, *Cladosterigma* Pat., *Mycogloea* L.S. Olive and *Tetracrium* Henn. and accepted four species in *Microcera*. In recent years, numerous newly-discovered species have been described by employing extensive sampling coupled with multigene phylogenies (Sung et al. 2007; Lombard and Crous 2012; Wei et al. 2019; Lucking et al. 2021). Lombard et al. (2015) performed a multi-gene phylogenetic analysis, using combined datasets of ITS, LSU, *tef1*- $\alpha$ , *act1*, *act*, *cmdA*, *his3*, *rpb1*, *rpb2* and *tub2* to clarify intraspecific and intergeneric relationships within Nectriaceae. In this paper, *M. pseudaulacaspidis* was distinguished from *M. kuwanaspidis* and established as a new species, based on base-pair differences, particularly in the LSU (17.67%), *tef1*- $\alpha$  (3.22%) and *his3* (3.82%). Additionally, *M. chrysomphaludis* formed a distinct and well-supported subclade and was found to be morphologically distinct from *M. coccophila* in terms of the size of asci, ascospores and macroconidia (Gräfenhan et al. 2011; O'Donnell et al. 2012; Dao et al. 2015). Through multigene phylogenetic analysis, the connection between the sexual and asexual morphs of *M. chrysomphaludis* was also confirmed.

Entomopathogenic fungi are common on scale insects and have great potential in biological control (Zha et al. 2019; Sharma et al. 2020). Based on field trials, *Microcera larvarum* has been reported to have a significant biological control effect of *Saissetia oleae*, an economically important pest of olive and citrus plants (Cozzi et al. 2002). *Microcera* species have also been exploited for various biopharmaceuticals in recent years due to their secondary metabolites with medicinal properties. For instance, parnafungins, extracted from *M. larvarum*, have intrinsic antifungal activity (Parish et al. 2008). Isaka et al. (2015) isolated two new ascochlorin derivatives from cultures of *Microcera* sp. BCC 17074 and demonstrated their significant cytotoxic activities against various cancer cells. Furthermore, Cadelis et al. (2020) isolated four new secondary metabolites from *M. larvarum* isolates, which exhibited potent antimicrobial activity.

This paper presents novel findings of two new entomopathogenic fungi, *Microcera chrysomphaludis* and *M. pseudaulacaspidis*, which were isolated from scale insects found on walnut trees in China. We conducted surveys in

numerous walnut orchards across Sichuan Province and observed significant infections of scale insects by these two species, resulting in high mortality rates, particularly in wet and humid conditions. Further screening and evaluation of these entomopathogenic fungi could facilitate their potential use as commercial biological control agents.

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## Additional information

### Conflict of interest

No conflict of interest was declared.

### Ethical statement

No ethical statement was reported.

### Funding

No funding was reported.

### Author contributions

Funding acquisition: CLY. Investigation: QZ, FHW, YD. Project administration: CLY. Supervision: XLX, CLY, YGL. Validation: RJ. Writing - review and editing: FL.

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### Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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